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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Bettina MOECKEL et al

SERIAL NO: New U.S. Application

FILED:

Herewith

FOR:

NUCLEOTIDE SEQUENCES WHICH CODE FOR THE rpoB GENE

REQUEST FOR PRIORITY

ASSISTANT COMMISSIONER FOR PATENTS WASHINGTON, D.C. 20231

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- □ Full benefit of the filing date of U.S. Application Serial Number, filed, is claimed pursuant to the provisions of 35 U.S.C. §120.
- ☐ Full benefit of the filing date of U.S. Provisional Application Serial Number, filed, is claimed pursuant to the provisions of 35 U.S.C. §119(e).
- Applicants claim any right to priority from any earlier filed applications to which they may be entitled pursuant to the provisions of 35 U.S.C. §119, as noted below.

In the matter of the above-identified application for patent, notice is hereby given that the applicants claim as priority:

COUNTRY

APPLICATION NUMBER

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Certified copies of the corresponding Convention Application(s)

- is submitted herewith
- □ will be submitted prior to payment of the Final Fee
- were filed in prior application Serial No. filed
- were submitted to the International Bureau in PCT Application Number.
 Receipt of the certified copies by the International Bureau in a timely manner under PCT Rule 17.1(a) has been acknowledged as evidenced by the attached PCT/IB/304.
- ☐ (A) Application Serial No.(s) were filed in prior application Serial No. filed; and
 - (B) Application Serial No.(s)
 - □ are submitted herewith
 - □ will be submitted prior to payment of the Final Fee

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Prioritätsbescheinigung über die Einreichung einer Patentanmeldung

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Bezeichnung:

Neue für das rpoB-Gen kodierende Nukleotidse-

quenzen

IPC:

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Bemerkung:

Die nachgereichte Seite 39 der Beschreibung ist am

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Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

München, den 29. Mai 2001 Deutsches Patent- und Markenamt Der Präsident

Inn Auftrag

Hiebinger

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Neue für das rpoB-Gen kodierende Nukleotidsequenzen

Gegenstand der Erfindung sind für das rpoB-Gen kodierende Nukleotidsequenzen aus coryneformen Bakterien und ein Verfahren zur fermentativen Herstellung von Aminosäuren unter Verwendung von Bakterien, in denen das rpoB-Gen verstärkt wird.

Stand der Technik

L-Aminosauren, insbesondere L-Lysin, finden in der Humanmedizin und in der pharmazeutischen Industrie, in der Lebensmittelindustrie und ganz besonders in der Tierernährung, Anwendung.

Es ist bekannt, daß Aminosäuren durch Fermentation von Stämmen coryneformer Bakterien, insbesondere Corynebacterium glutamicum, hergestellt werden. Wegen der großen Bedeutung wird ständig an der Verbesserung der Herstellverfahren gearbeitet. Verfahrensverbesserungen können fermentationstechnische Maßnahmen wie zum Beispiel Rührung und Versorgung mit Sauerstoff, oder die Zusammensetzung der Nährmedien wie zum Beispiel die Zuckerkonzentration während der Fermentation, oder die Aufarbeitung zur Produktform durch zum Beispiel Ionenaustauschchromatographie oder die intrinsischen Leistungseigenschaften des Mikroorganismus selbst betreffen.

Zur Verbesserung der Leistungseigenschaften dieser Mikroorganismen werden Methoden der Mutagenese, Selektion und Mutantenauswahl angewendet. Auf diese Weise erhält man Stämme, die resistent gegen Antimetabolite oder auxotroph für regulatorisch bedeutsame Metabolite sind und 30 Aminosäuren produzieren.

Seit einigen Jahren werden ebenfalls Methoden der rekombinanten DNA-Technik zur Stammverbesserung von

L-Aminosäure produzierenden Stämmen von Corynebacterium eingesetzt, indem man einzelne Aminosäure-Biosynthesegene amplifiziert und die Auswirkung auf die Aminosäure-Produktion untersucht.

5 Aufgabe der Erfindung

Die Erfinder haben sich zur Aufgabe gestellt, neue Maßnahmen zur verbesserten fermentativen Herstellung von Aminosäuren bereitzustellen.

Beschreibung der Erfindung

- Werden im folgenden L-Aminosäuren oder Aminosäuren erwähnt, sind damit eine oder mehrere Aminosäuren einschließlich ihrer Salze, ausgewählt aus der Gruppe L-Asparagin, L-Threonin, L-Serin, L-Glutamat, L-Glycin, L-Alanin, L-Cystein, L-Valin, L-Methionin, L-Isoleucin, L-Leucin, L-
- 15 Tyrosin, L-Phenylalanin, L-Histidin, L-Lysin, L-Tryptophan und L-Arginin gemeint. Besonders bevorzugt ist L-Lysin.

Wenn im folgenden L-Lysin oder Lysin erwähnt werden, sind damit nicht nur die Basen, sondern auch die Salze wie z.B. Lysin-Monohydrochlorid oder Lysin-Sulfat gemeint.

- Gegenstand der Erfindung ist ein isoliertes Polynukleotid aus coryneformen Bakterien, enthaltend eine für das rpoB-Gen kodierende Polynukleotidsequenz, ausgewählt aus der Gruppe
- a) Polynukleotid, das mindestens zu 70% identisch ist mit 25 einem Polynukleotid, das für ein Polypeptid kodiert, das die Aminosäuresequenz von SEQ ID No. 2 enthält,
 - b) Polynukleotid, das für ein Polypeptid kodiert, das eine Aminosäuresequenz enthält, die zu mindestens 70% identisch ist mit der Aminosäuresequenz von
- 30 SEQ ID No. 2,

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- c) Polynukleotid, das komplementär ist zu den Polynukleotiden von a) oder b), und
- d) Polynukleotid, enthaltend mindestens 15 aufeinanderfolgende Nukleotide der Polynukleotidsequenz von a), b) oder c),

wobei das Polypeptid bevorzugt die Aktivität der β -Untereinheit der RNA-Polymerase B aufweist.

Gegenstand der Erfindung ist ebenfalls das oben genannte Polynukleotid, wobei es sich bevorzugt um eine replizierbare DNA handelt, enthaltend:

- (i) die Nukleotidsequenz, gezeigt in SEQ ID No. 1, oder
- (ii) mindestens eine Sequenz, die der Sequenz (i) innerhalb des Bereichs der Degeneration des genetischen Kodes entspricht, oder
- (iii) mindestens eine Sequenz, die mit der zur Sequenz(i) oder (ii) komplementären Sequenzhybridisiert, und gegebenenfalls
- (iv) funktionsneutralen Sinnmutationen in (i), die die Aktivität des Proteins/Polypeptides nicht verändern

Ein weiterer Gegenstand der Erfindung sind schließlich Polynukleotide ausgewählt aus der Gruppe

- a) Polynukleotide enthaltend mindestens 15
 25 aufeinanderfolgende Nukleotide ausgewählt aus der
 Nukleotidsequenz von SEQ ID No. 1 zwischen den
 Positionen 1 und 701
 - b) Polynukleotide enthaltend mindestens 15 aufeinanderfolgende Nukleotide ausgewählt aus der

Nukleotidsequenz von SEQ ID No. 1 zwischen den Positionen 702 und 4199

c) Polynukleotide enthaltend mindestens 15 aufeinanderfolgende Nukleotide ausgewählt aus der Nukleotidsequenz von SEQ ID No. 1 zwischen den Positionen 4200 und 5099.

Weitere Gegenstände sind

- ein replizierbares Polynukleotid, insbesondere DNA, enthaltend die Nukleotidsequenz wie in SEQ ID No. 1 dargestellt;
- ein Polynukleotid, das für ein Polypeptid kodiert, das die Aminosäuresequenz, wie in SEQ ID No. 2 dargestellt, enthält;
- ein Vektor, enthaltend das erfindungsgemäße Polynukleotid, 15 insbesondere Pendelvektor oder Plasmidvektor, und
 - coryneforme Bakterien, die den Vektor enthalten oder in denen das rpoB-Gen verstärkt ist.

Gegenstand der Erfindung sind ebenso Polynukleotide, die im wesentlichen aus einer Polynukleotidsequenz bestehen, die erhältlich sind durch Screening mittels Hybridisierung einer entsprechenden Genbank eines coryneformen Bakteriums, die das vollständige Gen oder Teile davon enthält, mit einer Sonde, die die Sequenz des erfindungsgemäßen Polynukleotids gemäß SEQ ID No.1 oder ein Fragment davon enthält und Isolierung der genannten Polynukleotidsequenz.

Polynukleotide, die die Sequenzen gemäß der Erfindung enthalten, sind als Hybridisierungs-Sonden für RNA, cDNA und DNA geeignet, um Nukleinsäuren beziehungsweise Polynukleotide oder Gene in voller Länge zu isolieren, die für die β -Untereinheit der RNA-Polymerase B kodieren, oder um solche Nukleinsäuren beziehungsweise Polynukleotide oder

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Gene zu isolieren, die eine hohe Ähnlichkeit der Sequenz mit der des rpoB-Gens aufweisen. Sie sind ebenso zum Einbau in sogenannte "arrays", "micro arrays" oder "DNA chips" geeignet, um die entsprechenden Polynukleotide zu detektieren und zu bestimmen

Polynukleotide, die die Sequenzen gemäß der Erfindung enthalten, sind weiterhin als Primer geeignet, mit deren Hilfe mit der Polymerase-Kettenreaktion (PCR) DNA von Genen hergestellt werden kann, die für die β -Untereinheit der

10 RNA-Polymerase B kodieren.

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Solche als Sonden oder Primer dienende Oligonukleotide, enthalten mindestens 25, 26, 27, 28, 29 oder 30, bevorzugt mindestens 20, 21, 22, 23 oder 24, ganz besonders bevorzugt mindestens 15, 16, 17, 18 oder 19 aufeinanderfolgende

Nukleotide. Geeignet sind ebenfalls Oligonukleotide mit einer Länge von mindestens 31, 32, 33, 34, 35, 36, 37, 38, 39 oder 40, oder mindestens 41, 42, 43, 44, 45, 46, 47, 48, 49 oder 50 Nukleotiden. Gegebenenfalls sind auch Oligonukleotide mit einer Länge von mindestens 100, 150,

20 200, 250 oder 300 Nukleotiden geeignet.

"Isoliert" bedeutet aus seinem natürlichen Umfeld herausgetrennt.

"Polynukleotid" bezieht sich im allgemeinen auf Polyribonukleotide und Polydeoxyribonukleotide, wobei es 25 sich um nicht modifizierte RNA oder DNA oder modifizierte RNA oder DNA handeln kann.

Die Polynukleotide gemäß Erfindung schließen ein Polynukleotid gemäß SEQ ID No. 1 oder ein daraus hergestelltes Fragment und auch solche ein, die zu wenigstens besonders 70% bis 80%, bevorzugt zu wenigstens 81% bis 85%, besonders bevorzugt zu wenigstens 86% bis 90%, und ganz besonders bevorzugt zu wenigstens 91%, 93%, 95%,

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97% oder 99% identisch sind mit dem Polynukleotid gemäß SEQ ID No. 1 oder eines daraus hergestellten Fragmentes.

Unter "Polypeptiden" versteht man Peptide oder Proteine, die zwei oder mehr über Peptidbindungen verbundene Aminosäuren enthalten.

Die Polypeptide gemäß Erfindung schließen ein Polypeptid gemäß SEQ ID No. 2, insbesondere solche mit der biologischen Aktivität der β -Untereinheit der RNA-Polymerase B und auch solche ein, die zu wenigstens 70% bis 80%, bevorzugt zu wenigstens 81% bis 85%, besonders bevorzugt zu wenigstens 86% bis 90%, und ganz besonders bevorzugt zu wenigstens 91%, 93%, 95%, 97% oder 99% identisch sind mit dem Polypeptid gemäß SEQ ID No. 2 und die genannte Aktivität aufweisen.

- Die Erfindung betrifft weiterhin ein Verfahren zur fermermentativen Herstellung von Aminosäuren, ausgewählt aus der Gruppe L-Asparagin, L-Threonin, L-Serin, L-Glutamat, L-Glycin, L-Alanin, L-Cystein, L-Valin, L-Methionin, L-Isoleucin, L-Leucin, L-Tyrosin, L-
- Phenylalanin, L-Histidin, L-Lysin, L-Tryptophan und L-Arginin, unter Verwendung von coryneformen Bakterien, die insbesondere bereits Aminosäuren produzieren und in denen die für das rpoB-Gen kodierenden Nukleotidsequenzen verstärkt, insbesondere überexprimiert werden.
- Der Begriff "Verstärkung" beschreibt in diesem Zusammenhang die Erhöhung der intrazellulären Aktivität eines oder mehrerer Enzyme bzw. Proteine in einem Mikroorganismus, die durch die entsprechende DNA kodiert werden, indem man beispielsweise die Kopienzahl des Gens bzw. der Gene erhöht, einen starken Promotor verwendet oder ein Gen oder Allel verwendet, das für ein entsprechendes Enzym bzw. Protein mit einer hohen Aktivität kodiert und gegebenenfalls diese Maßnahmen kombiniert.

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Die Mikroorganismen, die Gegenstand der vorliegenden Erfindung sind, können L-Aminosäuren aus Glucose, Saccharose, Lactose, Fructose, Maltose, Melasse, Stärke, Cellulose oder aus Glycerin und Ethanol herstellen. Es kann sich um Vertreter coryneformer Bakterien insbesondere der Gattung Corynebacterium handeln. Bei der Gattung Corynebacterium ist insbesondere die Art Corynebacterium glutamicum zu nennen, die in der Fachwelt für ihre Fähigkeit bekannt ist, L-Aminosäuren zu produzieren.

Geeignete Stämme der Gattung Corynebacterium, insbesondere der Art Corynebacterium glutamicum (C. glutamicum), sind besonders die bekannten Wildtypstämme

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 und
Brevibacterium divaricatum ATCC14020

und daraus hergestellte L-Aminosäuren produzierende Mutanten bzw. Stämme, wie beispielsweise die L-Lysin produzierenden Stämme

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DG52-5
Corynebacterium glutamicum DSM5714 und
Corynebacterium glutamicum DSM12866.

Das neue, für die β -Untereinheit der RNA-Polymerase B kodierende rpoB-Gen von C. glutamicum wurde isoliert.

Zur Isolierung des rpoB-Gens oder auch anderer Gene von
C. glutamicum wird zunächst eine Genbank dieses

- Mikroorganismus in Escherichia coli (E. coli) angelegt.

 Das Anlegen von Genbanken ist in allgemein bekannten
 Lehrbüchern und Handbüchern niedergeschrieben. Als Beispiel
 seien das Lehrbuch von Winnacker: Gene und Klone, Eine
 Einführung in die Gentechnologie (Verlag Chemie, Weinheim,
- Deutschland, 1990), oder das Handbuch von Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) genannt. Eine sehr bekannte Genbank ist die des E. coli K-12 Stammes W3110, die von Kohara et al. (Cell 50, 495-508 (1987)) in λ -Vektoren angelegt wurde.
- Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) beschreiben eine Genbank von C. glutamicum ATCC13032, die mit Hilfe des Cosmidvektors SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) im E. coli K-12 Stamm NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575) angelegt wurde.
 - Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) wiederum beschreiben eine Genbank von C. glutamicum ATCC13032 unter Verwendung des Cosmids pHC79 (Hohn und Collins, Gene 11, 291-298 (1980)).
- Zur Herstellung einer Genbank von C. glutamicum in E. coli können auch Plasmide wie pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) oder pUC9 (Vieira et al., 1982, Gene, 19:259-268) verwendet werden. Als Wirte eignen sich besonders solche E. coli Stämme, die restriktions- und rekombinationsdefekt sind. Ein Beispiel hierfür ist der Stamm DH5amcr, der von Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) beschrieben wurde. Die mit Hilfe von Cosmiden klonierten langen DNA-Fragmente können anschließend wiederum in gängige, für die Sequenzierung geeignete Vektoren

subkloniert und anschließend sequenziert werden, so wie es z.B. bei Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977) beschrieben ist.

- Die erhaltenen DNA-Sequenzen können dann mit bekannten Algorithmen bzw. Sequenzanalyse-Programmen wie z.B. dem von Staden (Nucleic Acids Research 14, 217-232(1986)), dem von Marck (Nucleic Acids Research 16, 1829-1836 (1988)) oder dem GCG-Programm von Butler (Methods of Biochemical
- 10 Analysis 39, 74-97 (1998)) untersucht werden.

Die neue für das Gen rpoB kodierende DNA-Sequenz von C. glutamicum wurde gefunden, die als SEQ ID No. 1 Bestandteil der vorliegenden Erfindung ist. Weiterhin wurde aus der vorliegenden DNA-Sequenz mit den oben beschriebenen

- 15 Methoden die Aminosäuresequenz des entsprechenden Proteins abgeleitet. In SEQ ID No. 2 ist die sich ergebende Aminosäuresequenz des rpoB-Genproduktes dargestellt. Es ist bekannt, daß wirtseigene Enzyme die N-terminale Aminosäure Methionin bzw. Formylmethionin des gebildeten Proteins abspalten können.
 - Kodierende DNA-Sequenzen, die sich aus SEQ ID No. 1 durch die Degeneriertheit des genetischen Kodes ergeben, sind ebenfalls Bestandteil der Erfindung. In gleicher Weise sind DNA-Sequenzen, die mit SEQ ID No. 1 oder Teilen von SEQ ID
- No. 1 hybridisieren, Bestandteil der Erfindung. In der Fachwelt sind weiterhin konservative Aminosäureaustausche wie z.B. Austausch von Glycin gegen Alanin oder von Asparaginsäure gegen Glutaminsäure in Proteinen als "Sinnmutationen" ("sense mutations") bekannt, die zu keiner
- grundsätzlichen Veränderung der Aktivität des Proteins führen, d.h. funktionsneutral sind. Derartige Mutationen werden unter anderem auch als neutrale Substitutionen bezeichnet. Weiterhin ist bekannt, daß Änderungen am Nund/oder C-Terminus eines Proteins dessen Funktion nicht
- 35 wesentlich beeinträchtigen oder sogar stabilisieren können.

Angaben hierzu findet der Fachmann unter anderem bei Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), bei O'Regan et al. (Gene 77:237-251 (1989)), bei Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), bei Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) und in bekannten Lehrbüchern der Genetik und Molekularbiologie.

Aminosäuresequenzen, die sich in entsprechender Weise aus SEQ ID No. 2 ergeben, sind ebenfalls Bestandteil der Erfindung.

In gleicher Weise sind DNA-Sequenzen, die mit SEQ ID No. 1 oder Teilen von SEQ ID No. 1 hybridisieren Bestandteil der Erfindung. Schließlich sind DNA-Sequenzen Bestandteil der Erfindung, die durch die Polymerase-Kettenreaktion (PCR) unter Verwendung von Primern hergestellt werden, die sich aus SEQ ID No. 1 ergeben. Derartige Oligonukleotide haben typischerweise eine Länge von mindestens 15 Nukleotiden.

Anleitungen zur Identifizierung von DNA-Sequenzen mittels Hybridisierung findet der Fachmann unter anderem im Handbuch "The DIG System Users Guide for Filter 20 Hybridization" der Firma Boehringer Mannheim GmbH (Mannheim, Deutschland, 1993) und bei Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). Die Hybridisierung findet unter stringenten Bedingungen statt, das heisst, es werden nur Hybride gebildet, bei denen Sonde und Zielsequenz, d. h. die mit der Sonde behandelten Polynukleotide, mindestens 70% identisch sind. Es ist bekannt, dass die Stringenz der Hybridisierung einschließlich der Waschschritte durch Variieren der Pufferzusammensetzung, der Temperatur und der 30 Salzkonzentration beeinflußt bzw. bestimmt wird. Die Hybridisierungsreaktion wird vorzugsweise bei relativ niedriger Stringenz im Vergleich zu den Waschschritten durchgeführt (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

Für die Hybridisierungsreaktion kann beispielsweise ein 5x SSC-Puffer bei einer Temperatur von ca. 50°C – 68°C eingesetzt werden. Dabei können Sonden auch mit Polynukleotiden hybridisieren, die weniger als 70%

- Identität zur Sequenz der Sonde aufweisen. Solche Hybride sind weniger stabil und werden durch Waschen unter stringenten Bedingungen entfernt. Dies kann beispielsweise durch Senken der Salzkonzentration auf 2x SSC und gegebenenfalls nachfolgend 0,5x SSC (The DIG System User's
- Guide for Filter Hybridisation, Boehringer Mannheim,
 Mannheim, Deutschland, 1995) erreicht werden, wobei eine
 Temperatur von ca. 50°C 68°C eingestellt wird. Es ist
 gegebenenfalls möglich die Salzkonzentration bis auf 0,1x
 SSC zu senken. Durch schrittweise Erhöhung der
- Hybridisierungstemperatur in Schritten von ca. 1 2°C von 50°C auf 68°C können Polynukleotidfragmente isoliert werden, die beispielsweise mindestens 70% oder mindestens 80% oder mindestens 90% bis 95% Identität zur Sequenz der eingesetzten Sonde besitzen. Weitere Anleitungen zur
- Hybridisierung sind in Form sogenannter Kits am Markt erhältlich (z.B. DIG Easy Hyb von der Firma Roche Diagnostics GmbH, Mannheim, Deutschland, Catalog No. 1603558).
- Anleitungen zur Amplifikation von DNA-Sequenzen mit Hilfe der Polymerase-Kettenreaktion (PCR) findet der Fachmann unter anderem im Handbuch von Gait: Oligonukleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) und bei Newton und Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Deutschland, 1994).
 - 30 Es wurde gefunden, daß coryneforme Bakterien nach Verstärkung des rpoB-Gens in verbesserter Weise Aminosäuren produzieren.

Zur Erzielung einer Überexpression kann die Kopienzahl der entsprechenden Gene erhöht werden, oder es kann die

Promotor- und Regulationsregion oder die Ribosomenbindungsstelle, die sich stromaufwärts des Strukturgens befindet, mutiert werden. In gleicher Weise wirken Expressionskassetten, die stromaufwärts des

- 5 Strukturgens eingebaut werden. Durch induzierbare Promotoren ist es zusätzlich möglich, die Expression im Verlaufe der fermentativen Aminosäure-Produktion zu steigern. Durch Maßnahmen zur Verlängerung der Lebensdauer der m-RNA wird ebenfalls die Expression verbessert.
- 10 Weiterhin wird durch Verhinderung des Abbaus des Enzymproteins ebenfalls die Enzymaktivität verstärkt. Die Gene oder Genkonstrukte können entweder in Plasmiden mit unterschiedlicher Kopienzahl vorliegen oder im Chromosom integriert und amplifiziert sein. Alternativ kann weiterhin eine Überexpression der betreffenden Gene durch Veränderung
- der Medienzusammensetzung und Kulturführung erreicht werden.

Anleitungen hierzu findet der Fachmann unter anderem bei Martin et al. (Bio/Technology 5, 137-146 (1987)), bei Guerrero et al. (Gene 138, 35-41 (1994)). Tsuchiva und

- Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya und Morinaga (Bio/Technology 6, 428-430 (1988)), bei Eikmanns et al. (Gene 102, 93-98 (1991)), in der Europäischen Patentschrift 0 472 869, im US Patent 4,601,893, bei Schwarzer und Pühler (Bio/Technology 9, 84-87 (1991), bei
- Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), bei LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in der Patentanmeldung WO 96/15246, bei Malumbres et al. (Gene 134, 15 24 (1993)), in der japanischen Offenlegungsschrift
- JP-A-10-229891, bei Jensen und Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), bei Makrides (Microbiological Reviews 60:512-538 (1996)) und in bekannten Lehrbüchern der Genetik und Molekularbiologie.
- Zur Verstärkung wurde das erfindungsgemäße rpoB-Gen 35 beispielhaft mit Hilfe von episomalen Plasmiden

verwendet werden.

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überexprimiert. Als Plasmide eignen sich solche, die in coryneformen Bakterien repliziert werden. Zahlreiche bekannte Plasmidvektoren wie z.B. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) oder pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) beruhen auf den kryptischen Plasmiden pHM1519, pBL1 oder pGA1. Andere Plasmidvektoren wie z.B. solche, die auf pCG4 (US-A 4,489,160), oder pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), oder pAG1 (US-A 5,158,891) beruhen, können in gleicher Weise

Weiterhin eignen sich auch solche Plasmidvektoren mit Hilfe derer man das Verfahren der Genamplifikation durch

- Integration in das Chromosom anwenden kann, so wie es beispielsweise von Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) zur Duplikation bzw. Amplifikation des hom-thrB-Operons beschrieben wurde. Bei dieser Methode wird das vollständige
- Gen in einen Plasmidvektor kloniert, der in einem Wirt (typischerweise E. coli), nicht aber in C. glutamicum replizieren kann. Als Vektoren kommen beispielsweise pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob oder pK19mob (Schäfer et al., Gene 145, 69-73
- (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Firma Invitrogen, Groningen, Niederlande; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf
- et al, 1991, Journal of Bacteriology 173:4510-4516) oder pBGS8 (Spratt et al.,1986, Gene 41: 337-342) in Frage. Der Plasmidvektor, der das zu amplifizierende Gen enthält, wird anschließend durch Konjugation oder Transformation in den gewünschten Stamm von C. glutamicum überführt. Die Methode
- der Konjugation ist beispielsweise bei Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994))

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beschrieben. Methoden zur Transformation sind beispielsweise bei Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican und Shivnan (Bio/Technology 7, 1067-1070 (1989)) und Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)) beschrieben. Nach homologer Rekombination mittels eines "cross over"- Ereignisses enthält der resultierende Stamm mindestens zwei Kopien des betreffenden Gens.

- Es wurde weiterhin gefunden, dass der Austausch von

 10 Aminosäuren, insbesondere in den Abschnitten zwischen

 Position 1 bis 10, 190 bis 200 und 420 bis 450 in der

 Aminosäuresequenz der β-Untereinheit der RNA-Polymerase B

 dargestellt in SEQ ID No. 2, die Lysinproduktion

 coryneformer Bakterien verbessern.
- Es wurde auch gefunden, dass der Austausch von Aminosäuren an einer oder mehreren Positionen ausgewählt aus der Gruppe a) Position 1 bis 10, b) Position 190 bis 200 und c) Position 420 bis 450 in der SEQ ID No. 2 gleichzeitig erfolgen kann.
- In dem Bereich zwischen Position 1 bis 10 wird der Austausch von L-Prolin an der Position 5 gegen L-Leucin, L-Isoleucin oder L-Valin bevorzugt.
- In dem Bereich zwischen Position 190 bis 200 wird der Austausch von L-Serin an der Position 196 gegen L-
 - 25 Phenylalanin oder L-Tyrosin bevorzugt.

In dem Bereich zwischen 420 bis 450 werden folgende Austausche bevorzugt: Austausch von L-Leucin an der Position 424 gegen L-Prolin oder L-Arginin, Austausch von L-Serin an der Position 425 gegen L-Threonin oder L-Alanin, Austausch von L-Glutamin an Position 426 gegen L-Leucin oder L-Lysin, Austausch von L-Asparaginsäure an Position 429 gegen L-Isoleucin, L-Valin oder L-Leucin, Austausch von L-Histidin an Position 439 gegen jede andere proteinogene

Aminosäure ausgenommen L-Histidin, wird der Austausch von L-Serin an Position 444 gegen L-Leucin, L-Tyrosin oder L-Tryptophan und Austausch von L-Leucin an Position 446 gegen L-Prolin oder L-Isoleucin.

- Ganz besonders bevorzugt, werden ein oder mehrere
 Aminosäureaustausche ausgewählt aus der Gruppe: L-Prolin an
 Position 5 gegen L-Leucin, L-Serin an Position 196 gegen
 L-Phenylalanin, L-Aspartat an Position 429 gegen L-Valin
 und L-Histidin an Position 439 gegen L-Tyrosin.
- In SEQ ID No. 3 ist die Basensequenz des in Stamm DM1547 enthaltenen Allels rpoB-1547 dargestellt. Das rpoB-1547 Allel kodiert für ein Protein, dessen Aminosäuresequenz in SEQ ID No. 4 dargestellt ist. Das Protein enthält an Position 5 L-Leucin, an Position 196 L-Phenylalanin und an Position 429 L-Valin. Die DNA Sequenz des rpoB-1547 Allels (SEQ ID No. 3) enthält folgende Basenaustausche gegenüber dem rpoB Wildtypgen (SEQ ID No. 1): Thymin an Position 715 anstelle von Cytosin, Thymin an Position 1288 anstelle von Cytosin und Thymin an Position 1987 anstelle von Adenin.
 - In SEQ ID No. 5 ist die Basensequenz des in Stamm DM1546 enthaltenen Allels rpoB-1546 dargestellt. Das rpoB-1546 Allel kodiert für ein Protein, dessen Aminosäuresequenz in SEQ ID No. 6 dargestellt ist. Das Protein enthält an Position 439 L-Tyrosin. Die DNA Sequenz des rpoB-1546 Allels (SEQ ID No. 5) enthält folgende Basenaustausche gegenüber dem rpoB Wildtypgen (SEQ ID No. 1): Thymin an Position 2016 anstelle von Cytosin.

Für die Mutagenese können klassische Mutageneseverfahren unter Verwendung mutagener Stoffe wie beispielsweise N
Methyl-N'-Nitro-N-Nitrosoguanidin oder ultraviolettes Licht verwendet werden. Weiterhin können für die Mutagenese invitro Methoden wie beispielsweise eine Behandlung mit Hydroxylamin (Miller, J. H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia

coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) oder mutagene Oligonukleotide (T. A. Brown: Gentechnologie für Einsteiger, Spektrum Akademischer Verlag, Heidelberg, 1993) oder die Polymerasekettenreaktion (PCR), wie sie im Handbuch von Newton und Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994) beschrieben ist, verwendet werden.

Zusätzlich kann es für die Produktion von L-Aminosäuren
vorteilhaft sein, neben dem rpoB-Gen eines oder mehrere
Enzyme des jeweiligen Biosyntheseweges, der Glykolyse, der
Anaplerotik, des Zitronensäure-Zyklus, des PentosephosphatZyklus, des Aminosäure-Exports und gegebenenfalls
regulatorische Proteine zu verstärken, insbesondere
überzuexprimieren.

So kann für die Herstellung von L-Lysin zusätzlich zur Verstärkung des rpoB-Gens eines oder mehrere der Gene, ausgewählt aus der Gruppe

- das für die Dihydrodipicolinat-Synthase kodierende Gen dapA (EP-B 0 197 335),
 - das für die Glyceraldehyd-3-Phosphat-Dehydrogenase kodierende Gen gap (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- das für die Triosephosphat-Isomerase kodierende Gen tpi (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - das für die 3-Phosphoglycerat-Kinase kodierende Gen pgk
 (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - das für die Glucose-6-Phosphat-Dehydrogenase kodierende Gen zwf (JP-A-09224661),
- das für die Pyruvat-Carboxylase kodierende Gen pyc (DE-A-198 31 609),

- das für die Malat-Chinon-Oxidoreduktase kodierende Gen mgo (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- das für eine feed-back resistente Aspartatkinase
 kodierende Gen lysC (Kalinowski et al., Molecular Microbiologie 5(5), 1197-204 (1991)),
 - das für den Lysin-Export kodierende Gen lysE (DE-A-195 48 222),
 - das für das Zwal-Protein kodierende Gen zwal (DE: 19959328.0, DSM 13115), und
 - das für das ribosomale Protein S12 kodierende rpsL-Gen dargestellt in SEQ ID No. 7 und 8

verstärkt, insbesondere überexprimiert werden.

Der Begriff "Abschwächung" beschreibt in diesem

Zusammenhang die Verringerung oder Ausschaltung der intrazellulären Aktivität eines oder mehrerer Enzyme (Proteine) in einem Mikroorganismus, die durch die entsprechende DNA kodiert werden, indem man beispielsweise einen schwachen Promotor verwendet oder ein Gen bzw. Allel verwendet, das für ein entsprechendes Enzym mit einer niedrigen Aktivität kodiert bzw. das entsprechende Gen oder Enzym (Protein) inaktiviert und gegebenenfalls diese Maßnahmen kombiniert.

Weiterhin kann es für die Produktion von L-Aminosäuren 25 vorteilhaft sein, zusätzlich zur Verstärkung des rpoB-Gens eines oder mehrere Gene, ausgewählt aus der Gruppe

- das für die Phosphoenolpyruvat-Carboxykinase kodierende Gen pck (DE 199 50 409.1; DSM 13047),
- das für die Glucose-6-Phosphat-Isomerase kodierende Gen pgi (US 09/396,478; DSM 12969),

- o das für die Pyruvat-Oxidase kodierende Gen poxB (DE: 1995 1975.7; DSM 13114),
- das für das Zwa2-Protein kodierende Gen zwa2 (DE: 19959327.2, DSM 13113)
- 5 abzuschwächen, insbesondere die Expression zu verringern.

Weiterhin kann es für die Produktion von Aminosäuren vorteilhaft sein, neben der Verstärkung des rpoB-Gens unerwünschte Nebenreaktionen auszuschalten (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in:

Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

Die erfindungsgemäß hergestellten Mikroorganismen sind ebenfalls Gegenstand der Erfindung und können kontinuierlich oder diskontinuierlich im batch - Verfahren (Satzkultivierung) oder im fed batch (Zulaufverfahren) oder repeated fed batch Verfahren (repetitives Zulaufverfahren) zum Zwecke der Produktion von Aminosäuren kultiviert werden. Eine Zusammenfassung über bekannte Kultivierungsmethoden ist im Lehrbuch von Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) oder im Lehrbuch von Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)) beschrieben.

Das zu verwendende Kulturmedium muß in geeigneter Weise den Ansprüchen der jeweiligen Stämme genügen. Beschreibungen von Kulturmedien verschiedener Mikroorganismen sind im Handbuch "Manual of Methods for General Bacteriology" der American Society for Bacteriology (Washington D.C., USA, 1981) enthalten.

Als Kohlenstoffquelle können Zucker und Kohlehydrate wie z.B. Glucose, Saccharose, Lactose, Fructose, Maltose, Melasse, Stärke und Cellulose, Öle und Fette wie z.B. Sojaöl, Sonnenblumenöl, Erdnußöl und Kokosfett, Fettsäuren

wie z.B. Palmitinsäure, Stearinsäure und Linolsäure, Alkohole wie z.B. Glycerin und Ethanol und organische Säuren wie z.B. Essigsäure verwendet werden. Diese Stoffe können einzeln oder als Mischung verwendet werden.

Als Stickstoffquelle können organische Stickstoff-haltige Verbindungen wie Peptone, Hefeextrakt, Fleischextrakt, Malzextrakt, Maisquellwasser, Sojabohnenmehl und Harnstoff oder anorganische Verbindungen wie Ammoniumsulfat, Ammoniumchlorid, Ammoniumphosphat, Ammoniumcarbonat und Ammoniumnitrat verwendet werden. Die Stickstoffquellen können einzeln oder als Mischung verwendet werden.

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Als Phosphorquelle können Phosphorsäure, Kaliumdihydrogenphosphat oder Dikaliumhydrogenphosphat oder die
entsprechenden Natrium haltigen Salze verwendet werden. Das
Kulturmedium muß weiterhin Salze von Metallen enthalten wie
z.B. Magnesiumsulfat oder Eisensulfat, die für das Wachstum
notwendig sind. Schließlich können essentielle Wuchsstoffe
wie Aminosäuren und Vitamine zusätzlich zu den oben
genannten Stoffen eingesetzt werden. Dem Kulturmedium
können überdies geeignete Vorstufen zugesetzt werden. Die
genannten Einsatzstoffe können zur Kultur in Form eines
einmaligen Ansatzes hinzugegeben oder in geeigneter Weise
während der Kultivierung zugefüttert werden.

Zur pH-Kontrolle der Kultur werden basische Verbindungen
wie Natriumhydroxid, Kaliumhydroxid, Ammoniak bzw.
Ammoniakwasser oder saure Verbindungen wie Phosphorsäure
oder Schwefelsäure in geeigneter Weise eingesetzt. Zur
Kontrolle der Schaumentwicklung können Antischaummittel wie
z.B. Fettsäurepolyglykolester eingesetzt werden. Zur

Aufrechterhaltung der Stabilität von Plasmiden können dem
Medium geeignete selektiv wirkende Stoffe wie z.B.
Antibiotika hinzugefügt werden. Um aerobe Bedingungen
aufrechtzuerhalten, werden Sauerstoff oder Sauerstoff
haltige Gasmischungen wie z.B. Luft in die Kultur
eingetragen. Die Temperatur der Kultur liegt normalerweise

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bei 20°C bis 45°C und vorzugsweise bei 25°C bis 40°C. Die Kultur wird solange fortgesetzt, bis sich ein Maximum des gewünschten Produktes gebildet hat. Dieses Ziel wird normalerweise innerhalb von 10 Stunden bis 160 Stunden erreicht.

Methoden zur Bestimmung von L-Aminosäuren sind aus dem Stand der Technik bekannt. Die Analyse kann zum Beispiel so wie bei Spackman et al. (Analytical Chemistry, 30, (1958), 1190) beschrieben durch Ionenaustausch-Chromatographie mit anschließender Ninhydrin-Derivatisierung erfolgen, oder sie kann durch reversed phase HPLC erfolgen, so wie bei Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174) beschrieben.

Reinkulturen folgender Mikroorganismen wurden bei der

Deutschen Sammlung für Mikrorganismen und Zellkulturen

(DSMZ, Braunschweig, Deutschland)am 16. Januar 2001 gemäß

Budapester Vertrag hinterlegt:

- Corynebacterium glutamicum Stamm DM1546 als DSM 13993
- Corynebacterium glutamicum Stamm DM1547 als DSM 13994
- Das erfindungsgemäße Verfahren dient zur fermentativen Herstellung von Aminosäuren.

Die vorliegende Erfindung wird im folgenden anhand von Ausführungsbeispielen näher erläutert.

Die Isolierung von Plasmid-DNA aus Escherichia coli sowie
25 alle Techniken zur Restriktion, Klenow- und alkalische
Phosphatasebehandlung wurden nach Sambrook et al.
(Molecular Cloning. A Laboratory Manual (1989) Cold Spring
Harbour Laboratory Press, Cold Spring Harbor, NY, USA)
durchgeführt. Methoden zur Transformation von Escherichia
30 coli sind ebenfalls in diesem Handbuch beschrieben.

Die Zusammensetzung gängiger Nährmedien wie LB- oder TY-Medium kann ebenfalls dem Handbuch von Sambrook et al. entnommen werden.

Beispiel 1

5 Herstellung einer genomischen Cosmid-Genbank aus Corynebacterium glutamicum ATCC 13032

Chromosomale DNA aus Corynebacterium glutamicum ATCC 13032 wird wie bei Tauch et al. (1995, Plasmid 33:168-179) beschrieben isoliert und mit dem Restriktionsenzym Sau3AI (Amersham Pharmacia, Freiburg, Deutschland,

- 10 (Amersham Pharmacia, Freiburg, Deutschland, Produktbeschreibung Sau3AI, Code no. 27-0913-02) partiell gespalten. Die DNA-Fragmente werden mit shrimp alkalischer Phosphatase (Roche Diagnostics GmbH, Mannheim, Deutschland, Produktbeschreibung SAP, Code no. 1758250)
- dephosphoryliert. Die DNA des Cosmid-Vektors SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), bezogen von der Firma Stratagene (La Jolla, USA, Produktbeschreibung SuperCos1 Cosmid Vektor Kit, Code no. 251301) wird mit dem
- 20 Restriktionsenzym XbaI (Amersham Pharmacia, Freiburg, Deutschland, Produktbeschreibung XbaI, Code no. 27-0948-02) gespalten und ebenfalls mit shrimp alkalischer Phosphatase dephosphoryliert.
- Anschließend wird die Cosmid-DNA mit dem Restriktionsenzym
 BamHI (Amersham Pharmacia, Freiburg, Deutschland,
 Produktbeschreibung BamHI, Code no. 27-0868-04) gespalten.
 Die auf diese Weise behandelte Cosmid-DNA wird mit der
 behandelten ATCC13032-DNA gemischt und der Ansatz mit T4DNA-Ligase (Amersham Pharmacia, Freiburg, Deutschland,
 Produktbeschreibung T4-DNA-Ligase, Code no.27-0870-04)
 behandelt. Das Ligationsgemisch wird anschließend mit Hilfe
- behandelt. Das Ligationsgemisch wird anschließend mit Hilfe des Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Produktbeschreibung Gigapack II XL Packing Extract, Code no. 200217) in Phagen verpackt.

Zur Infektion des E. coli Stammes NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) werden die Zellen in 10 mM MgSO4 aufgenommen und mit einem Aliquot der Phagensuspension vermischt. Infektion und Titerung der Cosmidbank werden wie bei Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) beschrieben durchgeführt, wobei die Zellen auf LB-Agar (Lennox, 1955, Virology, 1:190) mit 100 mg/l Ampicillin ausplattiert werden. Nach Inkubation über Nacht bei 37°C werden rekombinante Einzelklone selektioniert.

Beispiel 2

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Germany).

Isolierung und Sequenzierung des rpoB-Gens

Die Cosmid-DNA einer Einzelkolonie wird mit dem Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden,

Germany) nach Herstellerangaben isoliert und mit dem Restriktionsenzym Sau3AI (Amersham Pharmacia, Freiburg, Deutschland, Produktbeschreibung Sau3AI, Product No. 27-0913-02) partiell gespalten. Die DNA-Fragmente werden mit shrimp alkalischer Phosphatase (Roche Diagnostics GmbH,

Mannheim, Deutschland, Produktbeschreibung SAP, Product No. 1758250) dephosphoryliert. Nach gelelektrophoretischer Auftrennung erfolgt die Isolierung der Cosmidfragmente im Größenbereich von 1500 bis 2000 bp mit dem QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden,

Die DNA des Sequenziervektors pZero-1, bezogen von der Firma Invitrogen (Groningen, Niederlande, Produktbeschreibung Zero Background Cloning Kit, Product No. K2500-01), wird mit dem Restriktionsenzym BamHI

(Amersham Pharmacia, Freiburg, Deutschland, Produktbeschreibung BamHI, Product No. 27-0868-04) gespalten. Die Ligation der Cosmidfragmente in den Sequenziervektor pZero-1 wird wie von Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring

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Harbor) beschrieben durchgeführt, wobei das DNA-Gemisch mit T4-Ligase (Pharmacia Biotech, Freiburg, Deutschland) über Nacht inkubiert wird. Dieses Ligationsgemisch wird anschließend in den E. coli Stamm DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) elektroporiert (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) und auf LB-Agar (Lennox, 1955, Virology, 1:190) mit 50 mg/l Zeocin ausplattiert.

Die Plasmidpräparation der rekombinanten Klone erfolgt mit dem Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Deutschland). Die Sequenzierung erfolgt nach der Dideoxy-Kettenabbruch-Methode von Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) mit Modifikationen nach Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). Es wird der "RR dRhodamin Terminator Cycle Sequencing Kit" von PE Applied Biosystems (Product No. 403044, Weiterstadt, Deutschland) verwendet. Die gelelektrophoretische Auftrennung und Analyse der Sequenzierreaktion erfolgt in einem "Rotiphorese NF Acrylamid/Bisacrylamid" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) mit dem "ABI Prism 377" Sequenziergerät von PE Applied Biosystems (Weiterstadt, Deutschland).

Die erhaltenen Roh-Sequenzdaten werden anschließend unter Anwendung des Staden-Programpakets (1986, Nucleic Acids Research, 14:217-231) Version 97-0 prozessiert. Die Einzelsequenzen der pZerol-Derivate werden zu einem zusammenhängenden Contig assembliert. Die computergestützte Kodierbereichsanalyse wird mit dem Programm XNIP (Staden, 1986, Nucleic Acids Research, 14:217-231) angefertigt.

Die erhaltene Nukleotidsequenz ist in SEQ ID No. 1 dargestellt. Die Analyse der Nukleotidsequenz ergibt ein offenes Leseraster von 3497 Basenpaaren, welches als rpoB-Gen bezeichnet wird. Das rpoB-Gen kodiert für ein Protein von 1165 Aminosäuren.

```
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50
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     Ile Leu Ala Val Ser Arg Gln Thr Lys Ser Val Val Asp Ile Pro Gly
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Ala Pro Gln Arg Tyr Ser Phe Ala Lys Val Ser Ala Pro Ile Glu Val

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5	ccc Pro	G] À ààà	cta Leu 40	Leu	gat Asp	ctt Leu	caa Gln	ctg Leu 45	gat Asp	tct Ser	tac Tyr	tcc Ser	tgg Trp 50	ctg Leu	att Ile	ggt Gly	860
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Patentansprüche

- Isoliertes Polynukleotid aus coryneformen Bakterien, enthaltend eine für das rpoB-Gen kodierende Polynukleotidsequenz, ausgewählt aus der Gruppe
- 5 a) Polynukleotid, das mindestens zu 70% identisch ist mit einem Polynukleotid, das für ein Polypeptid kodiert, das die Aminosäuresequenz von SEQ ID No. 2 enthält,
 - b) Polynukleotid, das für ein Polypeptid kodiert, das eine Aminosäuresequenz enthält, die zu mindestens 70% identisch ist mit der Aminosäuresequenz von SEQ ID No. 2,
 - c) Polynukleotid, das komplementär ist zu den Polynukleotiden von a) oder b), und
- d) Polynukleotid, enthaltend mindestens 15 aufeinanderfolgende Nukleotide der Polynukleotidsequenz von a), b) oder c)

wobei das Polypeptid bevorzugt die Aktivität der β -Untereinheit der RNA-Polymerase B aufweist.

- 20 2. Polynukleotid gemäß Anspruch 1, wobei das Polynukleotid eine in coryneformen Bakterien replizierbare, bevorzugt rekombinante DNA ist.
 - 3. Polynukleotid gemäß Anspruch 1, wobei das Polynukleotid eine RNA ist.
- Polynukleotid gemäß Anspruch 2, enthaltend die Nukleinsäuresequenz wie in SEQ ID No. 1 dargestellt.
 - 5. Replizierbare DNA gemäß Anspruch 2, enthaltend
 - (i) die Nukleotidsequenz, gezeigt in SEQ ID No. 1, oder

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- (ii) mindestens eine Sequenz, die der Sequenz(i) innerhalb des Bereichs der Degeneration des genetischen Kodes entspricht, oder
- (iii) mindestens eine Sequenz, die mit der zur Sequenz
 (i) oder (ii) komplementären Sequenz hybridisiert, und gegebenenfalls
 - (iv) funktionsneutrale Sinnmutationen in (i).
 - 6. Replizierbare DNA gemäß Anspruch 5, d a d u r c h g e k e n n z e i c h n e t, daß die Hybridisierung unter einer Stringenz entsprechend höchstens 2x SSC durchgeführt wird.
 - 7. Polynukleotidsequenz gemäß Anspruch 1, die für ein Polypeptid kodiert, das die in SEQ ID No. 2 dargestellte Aminosäuresequenz enthält.
- 15 8. Coryneforme Bakterien, in denen das rpoB-Gen verstärkt, insbesondere überexprimiert wird.
 - 9. Verfahren zur fermentativen Herstellung von L-Aminosäuren, insbesondere L-Lysin, dadurch gekennzeichnet, daß man folgende Schritte durchführt:
 - a) Fermentation der die gewünschte L-Aminosäure produzierenden coryneformen Bakterien, in denen man zumindest das rpoB-Gen oder dafür kodierende Nukleotidsequenzen verstärkt, insbesondere überexprimiert;
 - b) Anreicherung der L-Aminosäure im Medium oder in den Zellen der Bakterien, und
 - c) Isolieren der L-Aminosäure.
- 10. Verfahren gemäß Anspruch 9, dadurch gekennzeichnet, daß man Bakterien

einsetzt, in denen man zusätzlich weitere Gene des Biosyntheseweges der gewünschten L-Aminosäure verstärkt.

- 11. Verfahren gemäß Anspruch 9, d a d u r c h
 g e k e n n z e i c h n e t, daß man Bakterien
 einsetzt, in denen die Stoffwechselwege zumindest
 teilweise ausgeschaltet sind, die die Bildung der
 gewünschten L-Aminosäure verringern.
- 12. Verfahren gemäß Anspruch 9, d a d u r c h
 g e k e n n z e i c h n e t, daß man einen mit einem
 Plasmidvektor transformierten Stamm einsetzt, und der
 Plasmidvektor die für das rpoB-Gen kodierende
 Nukleotidsequenz trägt.
- 13. Verfahren gemäß Anspruch 9, d a d u r c h
 g e k e n n z e i c h n e t, daß man die Expression des
 (der) Polynukleotides (e), das (die) für das rpoB-Gen
 kodiert (kodieren) verstärkt, insbesondere
 überexprimiert.
- 14. Verfahren gemäß Anspruch 9, d a d u r c h
 g e k e n n z e i c h n e t, daß man die
 regulatorischen/katalytischen Eigenschaften des
 Polypetids (Enzymprotein) erhöht, für das das
 Polynukleotid rpoB kodiert.
- 15. Verfahren gemäß Anspruch 9, d a d u r c h g e k e n n z e i c h n e t, daß man zur Herstellung von L-Aminosäuren coryneforme Mikroorganismen fermentiert, in denen man gleichzeitig eines oder mehrere der Gene, ausgewählt aus der Gruppe
- das für die Dihydrodipicolinat-Synthase kodierende Gen dapA,
 - 15.2 das für die Glyceraldehyd-3-Phosphat-Dehydrogenase kodierende Gen gap,

- 15.3 das für die Triosephosphat-Isomerase kodierende Gen tpi, 15.4 das für die 3-Phosphoglycerat-Kinase kodierende Gen pgk, 5 15.5 das für die Glucose-6-Phosphat-Dehydrogenase kodierende Gen zwf, 15.6 das für die Pyruvat-Carboxylase kodierende Gen pyc, 15.7 das für die Malat-Chinon-Oxidoreduktase 10 kodierende Gen mgo, 15.8 das für eine feed-back resistente Aspartatkinase kodierende Gen lysC, 15.9 das für den Lysin-Export kodierende Gen lysE, 15.10 das für das Zwal-Protein kodierende Gen zwal 15 15.11 das für das ribosomale Protein S12 kodierende rpsL-Gen verstärkt bzw. überexprimiert. 16. Verfahren gemäß Anspruch 9, dadurch 20 gekennzeichnet, daß man zur Herstellung von L-Aminosäuren coryneforme Mikroorganismen
- fermentiert, in denen man gleichzeitig eines oder mehrere der Gene, ausgewählt aus der Gruppe

 16.1 das für die Phosphoenolpyruvat-Carboxykinase kodierende Gen pck,
 - 16.2 das für die Glucose-6-Phosphat Isomerase kodierende Gen pgi,
 - 16.3 das für die Pyruvat-Oxidase kodierende Gen poxB

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- 16.4 das für das Zwa2-Protein kodierende Gen zwa2 abschwächt.
- 17. Coryneforme Bakterien, die einen Vektor enthalten, der ein Polynukleotid gemäß Anspruch 1 trägt.
- 5 18. Verfahren gemäß einem oder mehreren der vorhergehenden Ansprüche, dad urch gekennzeichnet, daß man Mikroorganismen der Art Corynebacterium glutamicum einsetzt.
 - 19. Verfahren zum Auffinden von RNA, cDNA und DNA, um Nukleinsäuren, beziehungsweise Polynukleotide oder Gene zu isolieren, die für die β -Untereinheit der RNA-Polymerase B kodieren oder eine hohe Ähnlichkeit mit der Sequenz des rpoB-Gens aufweisen, d a d u r c h g e k e n n z e i c h n e t, daß man das Polynukleotid, enthaltend die Polynukleotidsequenzen gemäß den Ansprüchen 1, 2, 3 oder 4, als Hybridisierungssonden einsetzt.
 - 20. Verfahren gemäß Anspruch 18, d a d u r c h g e k e n n z e i c h n e t, daß man arrays, micro arrays oder DNA-chips einsetzt.
 - 21. Aus coryneformen Bakterien stammende DNA, kodierend für β-Untereinheiten der RNA-Polymerase B, wobei die zugehörigen Aminosäuresequenzen zwischen den Positionen 1 bis 10 in der SEQ ID No. 2 durch Aminosäureaustausch verändert sind.
 - 22. DNA gemäß Anspruch 21 d a d u r c h g e k e n n z e i c h n e t, daß diese für β-Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an Position 5 in der SEQ ID No. 2 L-Leucin, L-Isoleucin oder L-Valin enthalten.

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- 23. Aus coryneformen Bakterien stammende DNA, kodierend für β -Untereinheiten der RNA-Polymerase B, wobei die zugehörigen Aminosäuresequenzen zwischen den Positionen 190 bis 200 in der SEQ ID No. 2 durch Aminosäureaustausch verändert sind.
- 24. DNA gemäß Ansprüch 23 d a d u r c h g e k e n n z e i c h n e t, daß diese für β-Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an Position 196 in der SEQ ID No. 2 L-Phenylalanin oder L-Tyrosin enthalten.
- 25. Aus coryneformen Bakterien stammende DNA, kodierend für β -Untereinheiten der RNA-Polymerase B, wobei die zugehörigen Aminosäuresequenzen zwischen den Positionen 420 bis 450 in der SEQ ID No. 2 durch Aminosäureaustausch verändert sind.
- 26. DNA gemäß Anspruch 25 d a d u r c h g e k e n n z e i c h n e t, daß diese für die β-Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an den Positionen 439 in der SEQ ID No. 2 jede andere proteinogene Aminosäure ausgenommen L-Histidin enthalten.
- 27. DNA gemäß Anspruch 25 d a d u r c h
 g e k e n n z e i c h n e t, daß diese für die βUntereinheiten der RNA-Polymerase B kodieren, wobei die
 zugehörigen Aminosäuresequenzen an Position 424 in der
 SEQ ID No. 2 L-Prolin oder L-Arginin enthalten.
 - 28. DNA gemäß Anspruch 25 d a d u r c h g e k e n n z e i c h n e t, daß diese für die β-Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an Position 425 in der SEQ ID No. L-Threonin oder L-Alanin enthalten.
 - 29. DNA gemäß Anspruch 25 d a d u r c h g e k e n n z e i c h n e t, daß diese für die β-

Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an Position 426 in der SEQ ID No. 2 L-Leucin oder L-Lysin enthalten.

- 30. DNA gemäß Anspruch 25 d a d u r c h
 g e k e n n z e i c h n e t, daß diese für die βUntereinheiten der RNA-Polymerase B kodieren, wobei die
 zugehörigen Aminosäuresequenzen an Position 429 in der
 SEQ ID No. L-Isoleucin, L-Valin oder L-Leucin
 enthalten.
- 31. DNA gemäß Anspruch 25 d a d u r c h g e k e n n z e i c h n e t, daß diese für die β-Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an Position 444 in der SEQ ID No. 2 L-Leucin, L- Tyrosin oder L-Tryptophan enthalten.
 - 32. DNA gemäß Anspruch 25 d a d u r c h g e k e n n z e i c h n e t, daß diese für die β-Untereinheiten der RNA-Polymerase B kodieren, wobei die zugehörigen Aminosäuresequenzen an Position 446 in der SEQ ID No. 2 L-Prolin oder L-Isoleucin enthalten.
- 33. Aus coryneformen Bakterien stammende DNA, kodierend für β-Untereinheiten der RNA-Polymerase B, wobei die zugehörigen Aminosäuresequenzen an einer oder mehreren Positionen ausgewählt aus der Gruppe a) Position 1 bis 10, b) Position 190 bis 200 und c) Position 420 bis 450 in der SEQ ID No. 2 gleichzeitig durch Aminosäureaustausch verändert sind.
- 34. DNA gemäß Anspruch 33 d a d u r c h
 g e k e n n z e i c h n e t, daß diese für die β30 Untereinheiten der RNA-Polymerase B kodieren, wobei die
 zugehörigen Aminosäuresequenzen in der SEQ ID No. 2 an
 einer oder mehreren Positionen ausgewählt aus der
 Gruppe a) Position 5 L-Leucin, b) Position 196 L-

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Phenylalanin, c) Position 429 L-Valin, und d) Position 439 L-Tyrosin, enthalten.

- 35. DNA gemäß Anspruch 34 d a d u r c h g e k e n n z e i c h n e t, daß diese für die β-Untereinheit der RNA-Polymerase B kodiert, wobei die zugehörige Aminosäuresequenz an Position 5 L-Leucin, an Position 196 L-Phenylalanin, und an Position 429 L-Valin, enthält, dargestellt in SEQ ID No. 4.
- 36. DNA gemäß Anspruch 34 d a d u r c h
 g e k e n n z e i c h n e t, daß diese für die βUntereinheit der RNA-Polymerase B kodiert, wobei die
 zugehörigen Aminosäuresequenz an der Position 439 LTyrosin enthält, dargestellt in SEQ ID No. 6.
- 37. Aus coryneformen Bakterien stammende DNA, kodierend für die β -Untereinheit der RNA-Polymerase B, wobei die Basensequenz der DNA an der Position 715 Thymin enthält, an der Position 1288 Thymin enthält, und an der Position 1987 Thymin enthält, dargestellt in SEQ ID No. 3.
- 20 38. Aus coryneformen Bakterien stammende DNA, kodierend für die β -Untereinheit der RNA-Polymerase B, wobei die Basensequenz der DNA an der Position 2016 Thymin enthält, dargestellt in SEQ ID No. 5.

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Zusammenfassung

Die Erfindung betrifft ein isoliertes Polynukleotid, enthaltend eine Polynukleotidsequenz, ausgewählt aus der Gruppe

- 5 a) Polynukleotid, das mindestens zu 70% identisch ist mit einem Polynukleotid, das für ein Polypeptid kodiert, das die Aminosäuresequenz von SEQ ID No. 2 enthält,
 - b) Polynukleotid, das für ein Polypeptid kodiert, das eine Aminosäuresequenz enthält, die zu mindestens 70% identisch ist mit der Aminosäuresequenz von SEQ ID No. 2,
 - c) Polynukleotid, das komplementär ist zu den Polynukleotiden von a) oder b), und
- d) Polynukleotid, enthaltend mindestens 15
 aufeinanderfolgende Nukleotide der Polynukleotidsequenz von a), b) oder c),

und ein Verfahren zur fermentativen Herstellung von L-Aminosäuren unter Verwendung von coryneformen Bakterien, in denen zumindest das rpoB-Gen verstärkt vorliegt, und die Verwendung von Polynukleotiden, die die erfindungsgemäßen Sequenzen enthalten, als Hybridisierungssonden.



TRANSLATOR'S DECLARATION

I, Judith Atkinson, BA(Hons.), MITI., translator to Messrs. Taylor & Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of pages of a German Patent application in the German language with the title:

Neue für das rpoB-Gen kodierende Nukleotidsequenzen

identified by the code number 000781 BT at the upper left of each page and corresponding to client/matter number of the law firm of

and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

Date:	13th March 2001		By:	J.M. Athinson.
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Novel nucleotide sequences coding for the rpoB gene

The invention provides nucleotide sequences from coryneform bacteria coding for the rpoB gene, and a process for the production of amino acids by fermentation using bacteria in which the rpoB gene is enhanced.

Prior art

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L-amino acids, especially L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and, very especially, in the feeding of animals.

It is known that amino acids are produced by fermentation of strains of coryneform bacteria, especially Corynebacterium glutamicum. Because of their great importance, attempts are continuously being made to improve the production processes. Improvements to the processes may concern measures relating to the fermentation, such as, for example, stirring and oxygen supply, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or working up to the product form by, for example, ion-exchange chromatography, or the intrinsic performance properties of the microorganism itself.

In order to improve the performance properties of such microorganisms, methods of mutagenesis, selection and mutant selection are employed. Such methods yield strains which are resistant to antimetabolites or are auxotrophic for metabolites that are important in terms of regulation, and which produce amino acids.

For a number of years, methods of recombinant DNA technology have also been used for improving the strain of L-amino acid-producing strains of Corynebacterium, by

amplifying individual amino acid biosynthesis genes and studying the effect on amino acid production.

Object of the invention

The inventors have set themselves the object of providing novel measures for the improved production of amino acids by fermentation.

Description of the invention

Where L-amino acids or amino acids are mentioned hereinbelow, they are to be understood as meaning one or more amino acids, including their salts, selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-lysine is especially preferred.

Where L-lysine or lysine is mentioned hereinbelow, it is to be understood as meaning not only the bases but also the salts, such as, for example, lysine monohydrochloride or lysine sulfate.

- The invention provides an isolated polynucleotide from coryneform bacteria, containing a polynucleotide sequence coding for the rpoB gene, selected from the group
 - a) polynucleotide that is at least 70% identical with a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70% identical with the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide that is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably exhibiting the activity of the β -subunit of RNA polymerase B.

The invention also provides the above-mentioned polynucleotide, it preferably being a replicatable DNA containing:

- (i) the nucleotide sequence shown in SEQ ID No. 1, or
- (ii) at least one sequence that corresponds to sequence(i) within the region of the degeneracy of the qenetic code, or
 - (iii) at least one sequence that hybridizes with the sequence that is complementary to sequence (i) or (ii), and optionally
 - (iv) sense mutations in (i) which are neutral in terms of function and which do not change the activity of the protein/polypeptide.

Finally, the invention also provides polynucleotides selected from the group

- a) polynucleotides containing at least 15 consecutive nucleotides selected from the nucleotide sequence of SEQ ID No. 1 between positions 1 and 701
- b) polynucleotides containing at least 15 consecutive nucleotides selected from the nucleotide sequence of SEQ ID No. 1 between positions 702 and 4199
 - c) polynucleotides containing at least 15 consecutive nucleotides selected from the nucleotide sequence of SEQ ID No. 1 between positions 4200 and 5099.

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The invention also provides

- a replicatable polynucleotide, especially DNA, containing the nucleotide sequence as shown in SEQ ID No. 1;
- a polynucleotide that codes for a polypeptide containing the amino acid sequence as shown in SEQ ID No. 2;
 - a vector containing the polynucleotide of the invention, especially a shuttle vector or a plasmid vector, and
 - coryneform bacteria which contain the vector or in which the rpoB gene has been enhanced.
- The invention also provides polynucleotides consisting substantially of a polynucleotide sequence, which are obtainable by screening, by means of hybridization, a corresponding gene library of a coryneform bacteria that contains the complete gene or parts thereof, using a probe containing the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and isolating the mentioned polynucleotide sequence.
 - Polynucleotides that contain the sequences of the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate in their complete length nucleic acids or polynucleotides or genes that code for the β -subunit of RNA polymerase B, or in order to isolate nucleic acids or polynucleotides or genes that are very similar to the sequence of the rpoB gene. They are likewise suitable for incorporation into so-called "arrays", "micro arrays" or "DNA chips" in order to detect and determine the corresponding polynucleotides.
 - Polynucleotides that contain the sequences of the invention are also suitable as primers, with the aid of which it is possible, by means of the polymerase chain reaction (PCR), to produce DNA of genes that code for the β -subunit of RNA polymerase B.

Such oligonucleotides acting as probes or primers contain at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very especially preferably at least 15, 16, 17, 18 or 19, consecutive nucleotides. Also suitable are oligonucleotides having a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or of at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides. Oligonucleotides having a length of at least 100, 150, 200, 250 or 300 nucleotides may also be suitable.

10 "Isolated" means removed from its natural environment.

"Polynucleotide" generally refers to polyribonucleotides and polydeoxyribonucleotides, it being possible for the RNA or DNA to be unmodified or modified.

The polynucleotides of the invention include a

polynucleotide according to SEQ ID No. 1 or a fragment
prepared therefrom, and also polynucleotides that are at
least especially from 70% to 80%, preferably at least from
81% to 85%, especially preferably at least from 86% to 90%,
and very especially preferably at least 91%, 93%, 95%, 97%

or 99%, identical with the polynucleotide according to SEQ
ID No. 1, or with a fragment prepared therefrom.

"Polypeptides" are to be understood as being peptides or proteins that contain two or more amino acids bonded *via* peptide bonds.

The polypeptides of the invention include a polypeptide according to SEQ ID No. 2, especially those having the biological activity of the β-subunit of RNA polymerase B, and also those that are at least from 70% to 80%, preferably at least from 81% to 85%, especially preferably at least from 86% to 90%, and very especially preferably at least 91%, 93%, 95%, 97% or 99%, identical with the polypeptide according to SEQ ID No. 2 and exhibit the mentioned activity.

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The invention also provides a process for the production, by fermentation, of amino acids selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine, using coryneform bacteria which, in particular, already produce amino acids and in which the nucleotide sequences coding for the rpoB gene are enhanced, especially overexpressed.

The term "enhancement" in this connection describes the increasing of the intracellular activity of one or more enzymes or proteins in a microorganism that are coded for by the corresponding DNA, by, for example, increasing the number of copies of the gene or genes, using a strong promoter or using a gene or allele that codes for a corresponding enzyme or protein having a high level of activity, and optionally by combining those measures.

The microorganisms provided by the present invention can produce L-amino acids from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They may be representatives of coryneform bacteria, especially of the genus Corynebacterium. In the case of the genus Corynebacterium, special mention may be made of the species Corynebacterium glutamicum, which is known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, especially of the species Corynebacterium glutamicum (C. glutamicum), are especially the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965

Brevibacterium flavum ATCC14067 Brevibacterium lactofermentum ATCC13869 and Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared
therefrom, such as, for example, the L-lysine-producing
strains

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712

10 Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DG52-5
Corynebacterium glutamicum DSM5714 and
Corynebacterium glutamicum DSM12866.

The new rpoB gene of C. glutamicum coding for the β -subunit of RNA polymerase B has been isolated.

In order to isolate the rpoB gene or other genes from C. glutamicum, a gene library of that microorganism in Escherichia coli (E. coli) is first prepared. The 20 preparation of gene libraries is written down in generally known textbooks and handbooks. There may be mentioned as an example the textbook of Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the handbook of Sambrook et al.: 25 Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A very well known gene library is that of the E. coli K-12 strain W3110, which has been prepared by Kohara et al. (Cell 50, 495-508 (1987)) in λ vectors. Bathe et al. (Molecular and General Genetics, 30 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032, which has been prepared with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the

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E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (sic) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)).

For the preparation of a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are 10 especially those E. coli strains that are restriction- and recombination-defective. An example thereof is the strain DH5cmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the 15 aid of cosmids can then in turn be subcloned into customary vectors suitable for sequencing and then sequenced, as is described, for example, in Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977). 20

The resulting DNA sequences can then be studied using known algorithms or sequence-analysis programs, such as, for example, that of Staden (Nucleic Acids Research 14, 217-232 (1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The novel DNA sequence of C. glutamicum coding for the rpoB gene has been found and, as SEQ ID No. 1, forms part of the present invention. Furthermore, the amino acid sequence of the corresponding protein has been derived from the present DNA sequence using the methods described above. The resulting amino acid sequence of the rpoB gene product is shown in SEQ ID No. 2. It is known that enzymes belonging to the host are able to cleave the N-terminal amino acid

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methionine or formylmethionine of the protein that is formed.

Coding DNA sequences that result from SEQ ID No. 1 by the degeneracy of the genetic code also form part of the invention. Likewise, DNA sequences that hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 form part of the invention. Furthermore, to those skilled in the art, conservative amino acid substitutions, such as, for example, the substitution of glycine with alanine or of aspartic acid with glutamic acid, in proteins are known as 10 sense mutations, which do not lead to any fundamental change in the activity of the protein, that is to say are neutral in terms of function. Such mutations are known inter alia also as neutral substitutions. It is also known that changes at the N- and/or C-terminus of a protein do 15 not substantially impair its function or may even stabilise it. The person skilled in the art will find relevant information inter alia in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 20 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences that result in a corresponding manner from SEQ ID No. 2 likewise form part 25 of the invention.

Similarly, DNA sequences that hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 form part of the invention. Finally, DNA sequences that are produced by the polymerase chain reaction (PCR) using primers that result from SEQ ID No. 1 form part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

The person skilled in the art will find instructions on the identification of DNA sequences by means of hybridization inter alia in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH

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(Mannheim, Germany, 1993) and in Liebl et al.

(International Journal of Systematic Bacteriology (1991)

41: 255-260). The hybridization takes place under stringent conditions, that is to say there are formed only hybrids in which the probe and the target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out with relatively low stringency as compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

There may be used for the hybridization reaction, for example, a 5x SSC buffer at a temperature of approximately 15 from 50°C to 68°C. In that case, probes may also hybridize with polynucleotides that are less than 70% identical with the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. That may be achieved, for example, by lowering the salt 20 concentration to 2x SSC and optionally subsequently to 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), a temperature of approximately from 50°C to 68°C being set. It is optionally possible to lower the salt concentration 25 down to 0.1x SSC. By raising the hybridization temperature stepwise from 50°C to 68°C in steps of approximately from 1 to 2°C, it is possible to isolate polynucleotide fragments that are, for example, at least 70% or at least 80% or at least from 90% to 95% identical with the sequence of the 30 probe used. Further instructions for hybridization are commercially available in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1603558).

The person skilled in the art will find instructions on the amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) inter alia in the handbook of Gait: Oligonukleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids in an improved manner after enhancement of the rpoB gene.

In order to achieve overexpression, the number of copies of 10 the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site, which is located upstream of the structural gene, can be mutated. Expression cassettes inserted upstream of the structural gene have a similar effect. By means of inducible promoters 15 it is additionally possible to increase the expression in the course of the production of amino acids by fermentation. Expression is also improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing degradation of the 20 enzyme protein. The genes or gene constructs may either be present in plasmids with different numbers of copies or be integrated and amplified in the chromosome. Alternatively, overexpression of the genes in question may also be achieved by changing the composition of the medium and the 25 manner in which culturing is carried out.

The person skilled in the art will find instructions thereon in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)),

30 Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European patent specification 0 472 869, in US patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of

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Bacteriology 175, 1001-1007 (1993)), in patent application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese Offenlegungsschrift JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

For the purposes of enhancement, the rpoB gene of the invention was overexpressed, for example, with the aid of episomal plasmids. Suitable plasmids are those which are 10 replicated in coryneform bacteria. Many known plasmid vectors, such as, for example, pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)), are based on the cryptic 15 plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as, for example, those which are based on pCG4 (US-A 4,489,160) or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)) or pAG1 (US-A 5,158,891), may likewise be used. 20

Also suitable are those plasmid vectors with the aid of which the process of gene amplification by integration into the chromosome can be applied, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for the duplication or 25 amplification of the hom-thrB operon. In that method, the complete gene is cloned into a plasmid vector that is able to replicate in a host (typically E. coli), but not in C. glutamicum. Suitable vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob 30 or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-32684; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard et al., Journal of 35

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Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al., 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector containing the gene to be amplified is then transferred to the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods of transformation are described, for example, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" occurrence, the resulting strain contains at least two copies of the gene in question. 15

It has also been found that the substitution of amino acids, especially in the sections between position 1 to 10, 190 to 200 and 420 to 450 in the amino acid sequence of the $\beta\text{-subunit}$ of RNA polymerase B shown in SEQ ID No. 2, improves the lysine production of coryneform bacteria.

It has also been found that the substitution of amino acids at one or more positions selected from the group a) position 1 to 10, b) position 190 to 200 and c) position 420 to 450 in SEQ ID No. 2 may take place simultaneously.

In the region between position 1 to 10, preference is given to the substitution of L-proline at position 5 by Lleucine, L-isoleucine or L-valine.

In the region between position 190 to 200, preference is given to the substitution of L-serine at position 196 by Lphenylalanine or L-tyrosine.

In the region between 420 to 450, the following substitutions are preferred: substitution of L-leucine at position 424 by L-proline or L-arginine, substitution of

L-serine at position 425 by L-threonine or L-alanine, substitution of L-glutamine at position 426 by L-leucine or L-lysine, substitution of L-aspartic acid at position 429 by L-isoleucine, L-valine or L-leucine, substitution of L-histidine at position 439 by any proteinogenic amino acid with the exception of L-histidine, is (sic) the substitution of L-serine at position 444 by L-leucine, L-tyrosine or L-tryptophan, and substitution of L-leucine at position 446 by L-proline or L-isoleucine.

- 10 Very special preference is given to one or more amino acid substitutions selected from the group: L-proline at position 5 by L-leucine, L-serine at position 196 by L-phenylalanine, L-aspartate at position 429 by L-valine, and L-histidine at position 439 by L-tyrosine.
- 15 SEQ ID No. 3 shows the base sequence of the allele rpoB-1547 contained in strain DM1547. The rpoB-1547 allele codes for a protein the amino acid sequence of which is shown in SEQ ID No. 4. The protein contains L-leucine at position 5, L-phenylalanine at position 196 and L-valine at position
- 20 429. The DNA sequence of the rpoB-1547 allele (SEQ ID No. 3) contains the following base substitutions as compared with the rpoB wild-type gene (SEQ ID No. 1): thymine at position 715 instead of cytosine, thymine at position 1288 instead of cytosine, and thymine at position 1987 instead of adenine.

SEQ ID No. 5 shows the base sequence of the allele rpoB1546 contained in strain DM1546. The rpoB-1546 allele codes
for a protein the amino acid sequence of which is shown in
SEQ ID No. 6. The protein contains L-tyrosine at position
30 439. The DNA sequence of the rpoB-1546 allele (SEQ ID
No. 5) contains the following base substitutions as
compared with the rpoB wild-type gene (SEQ ID No. 1):
thymine at position 2016 instead of cytosine.

There may be employed for the mutagenesis conventional methods of mutagenesis using mutagenic substances such as, for example, N-methyl-N'-nitro-N-nitrosoguanidine or ultraviolet light. There may also be used for the mutagenesis in vitro methods such as, for example, treatment with hydroxylamine (Miller, J. H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T. A. Brown: Gentechnologie für Einsteiger, Spektrum Akademischer Verlag, Heidelberg, 1993) or the polymerase chain reaction (PCR), as is described in the handbook of Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994).

In addition, it may be advantageous for the production of L-amino acids to enhance, especially to overexpress, in addition to the rpoB gene, one or more enzymes of the biosynthesis pathway in question, of glycolysis, of the anaplerotic pathway, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export, and, optionally, regulatory proteins.

Accordingly, for the production of L-lysine, in addition to enhancing the rpoB gene, one or more genes selected from the group

- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
 - the gene gap coding for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene tpi coding for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - the gene pgk coding for 3-phosphoglycerate kinase
 (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),
- the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609),
- o the gene mgo coding for malate quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
 - the gene lysC coding for a feed-back resistant aspartate kinase (Kalinowski et al., Molecular Microbiologie 5(5), 1197-1204 (1991)),
 - the gene lysE coding for lysine export (DE-A-195 48 222),
 - the gene zwal coding for the Zwal protein (DE: 19959328.0, DSM 13115), and
- the rpsL gene coding for ribosomal protein S12 and shown in SEQ ID No. 7 and 8

may be enhanced, especially overexpressed.

The term "attenuation" in this connection describes the diminution or exclusion of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded for by the corresponding DNA, by, for example, using a weak promoter or using a gene or allele that codes for a corresponding enzyme having low activity, or by inactivating the corresponding gene or enzyme (protein), and optionally by combining those measures.

25 Furthermore, it may be advantageous for the production of L-amino acids, in addition to enhancing the rpoB gene, to attenuate, especially to diminish the expression of, one or more genes selected from the group

- the gene pck coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the gene pgi coding for glucose-6-phosphate isomerase
 (US 09/396,478; DSM 12969),
- 5 the gene poxB coding for pyruvate oxidase (DE: 1995 1975.7; DSM 13114),
 - the gene zwa2 coding for the Zwa2 protein (DE: 19959327.2, DSM 13113).
- It may also be advantageous for the production of amino
 acids, in addition to enhancing the rpoB gene, to exclude
 undesired secondary reactions (Nakayama: "Breeding of Amino
 Acid Producing Micro-organisms", in: Overproduction of
 Microbial Products, Krumphanzl, Sikyta, Vanek (eds.),
 Academic Press, London, UK, 1982).
- The microorganisms produced according to the invention also form part of the invention and can be cultivated, for the purposes of the production of amino acids, continuously or discontinuously in the batch, fed batch or repeated fed batch process. A summary of known cultivation methods is described in the textbook of Chmiel (Bioprozeβtechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook of Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).
- The culture medium to be used must meet the requirements of the strains in question in a suitable manner. Descriptions of culture media for various microorganisms are to be found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology

 (Washington D.C., USA, 1981).

There may be used as the carbon source sugars and carbohydrates, such as, for example, glucose, saccharose,

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lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soybean oil, sunflower oil, groundnut oil and coconut oil, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid. Those substances may be used individually or in the form of a mixture.

There may be used as the nitrogen source organic nitrogencontaining compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or in the form of a mixture.

There may be used as the phosphorus source phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts. The culture medium must also contain salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, may be used in addition to the above-mentioned substances. Suitable precursors may also be added to the culture medium. The mentioned substances may be added to the culture in the form of a single batch, or they may be fed in in a suitable manner during the cultivation.

In order to control the pH value of the culture, basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acid compounds, such as phosphoric acid or sulfuric acid, are expediently used. In order to control the development of foam, anti-foams, such as, for example, fatty acid polyglycol esters, may be used. In order to maintain the stability of plasmids, suitable substances having a selective action, such as, for example,

antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or gas mixtures containing oxygen, such as, for example, air, are introduced into the culture. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. The culture is continued until the maximum amount of the desired product has formed. That aim is normally achieved within a period of from 10 hours to 160 hours.

Methods of determining L-amino acids are known from the
prior art. The analysis may be carried out, for example, as
described in Spackman et al. (Analytical Chemistry, 30,
(1958), 1190) by ion-exchange chromatography with
subsequent ninhydrin derivatization, or it may be carried
out by reversed phase HPLC, as described in Lindroth et al.
(Analytical Chemistry (1979) 51: 1167-1174).

Pure cultures of the following microorganisms were deposited on 16 January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty:

- 20 ∘ Corynebacterium glutamicum strain DM1546 as DSM 13993
 - o Corynebacterium glutamicum strain DM1547 as DSM 13994.

The process of the invention is used for the production of amino acids by fermentation.

The present invention is explained in greater detail below by means of Examples.

The isolation of plasmid DNA from Escherichia coli and all techniques for restriction, Klenow and alkaline phosphatase treatment were carried out according to Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for the transformation of Escherichia coli are also described in that handbook.

The composition of common nutrient media, such as LB or TY medium, will also be found in the handbook of Sambrook et al..

Example 1

5 Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 is isolated as described in Tauch et al. (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, 10 product description Sau3AI, Code no. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Code no. 1758250). The DNA of cosmid vector SuperCosl (Wahl et al. (1987) Proceedings of 15 the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vektor Kit, Code no. 251301), is cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, 20 Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA is then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Code no. 27-0868-04). The cosmid DNA so treated is mixed with the treated ATCC13032 DNA, and the batch is treated with T4-DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA ligase, Code no. 27-0870-04). The ligation mixture is then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, Code no. 200217).

For infection of E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575), the cells are taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. Infection and titration of the cosmid library are carried out as described in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones are selected.

Example 2

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Isolation and sequencing of the rpoB gene

The cosmid DNA of an individual colony is isolated using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) according to the manufacturer's instructions, and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Product No. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250). After separation by gel electrophoresis, cosmid fragments having a size in the range from 1500 to 2000 bp are isolated using the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of sequencing vector pZero-1, obtained from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, Product No. K2500-01), is cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Product No. 27-0868-04). Ligation of the cosmid fragments into the sequencing vector pZero-1 is carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture

being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). The ligation mixture is then electroporated into E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-347) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l Zeocin.

Plasmid preparation of the recombinant clones is carried out using the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). Sequencing is effected by the dideoxy 10 chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied 15 Biosystems (Product No. 403044, Weiterstadt, Germany) is used. Separation by gel electrophoresis and analysis of the sequencing reaction is carried out in a "Rotiphorese NF Acrylamid/Bisacrylamid" gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" 20 sequencing device from PE Applied Biosystems (Weiterstadt, Germany).

The resulting crude sequence data are then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) Version 97-0. The individual sequences of the pZero1 derivatives are assembled to a coherent contig. The computer-assisted coding region analysis is prepared using the program XNIP (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence gives an open reading frame of 3497 base pairs, which is designated the rpoB gene. The rpoB gene codes for a protein of 1165 amino acids.

SEQUENCE LISTING

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5099

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	Ala	Glu 610	Ala	Pro	Phe	Val	Gly 615	Thr	Gly	Met	Glu	Gln 620	Arg	Ala	Ala	Tyr
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	_				725					730					Leu 735	
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	Leu	Ala 850	Pro	Gly	Val	Asn	Glu 855	Met	Ile	Arg	Ile	Tyr 860	Val	Ala	Gln	Lys
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Gly Ile Pro Glu Ser Phe Lys Val Leu Leu Lys Glu Leu Gln Ser Leu 1115 105 1110 Cys Leu Asn Val Glu Val Leu Ser Ala Asp Gly Thr Pro Met Glu Leu 5 1125 1130 Ala Gly Asp Asp Asp Phe Asp Gln Ala Gly Ala Ser Leu Gly Ile 1140 1145 10 Asn Leu Ser Arg Asp Glu Arg Ser Asp Ala Asp Thr Ala 1155 1160 15 <210> 3 <211> 5099 <212> DNA <213> Corynebacterium glutamicum 20 <220> <221> CDS <222> (702)..(4196) <223> rpoB gene allele 1547 25 <400> 3 acaatgtgac tcgtgatttt tgggtggatc agcgtaccgg tttggttgtc gatctagctg 60 aaaatattga tgatttttac ggcgaccgca gcggccagaa gtacgaacag aaattgcttt 120 30 tcgacgcctc cctcgacgat gcagctgtct ctaagctggt tgcacaggcc gaaagcatcc 180 ctgatggaga tgtgagcaaa atcgcaaata ccgtaggtat tgtgatcggt gcggtattgg 240 ctctcgtggg cctggccggg tgttttgggg cgtttgggaa gaaacgtcga gaagcttaac 300 35 ctgctgttca aatagatttt ccctgtttcg aattgcggaa accccgggtt tgtttgctag 360 ggtgcctcgt agaaggggtc aagaagattt ctgggaaacg cgcccgtgcg gttggttgct 420 40 aatagcacgc ggagcaccag atgaaaaatc tcccctttac tttcgcgcgc gattggtata 480 ctctgagtcg ttgcgttgga attcgtgact ctttttcgtt cctgtagcgc caagaccttg 540 atcaaggtgg tttaaaaaaa ccgatttgac aaggtcattc agtgctatct ggagtcgttc 600 45 agggggatcg ggttcctcag cagaccaatt gctcaaaaat accagcggtg ttgatctgca 660 cttaatggcc ttgaccagcc aggtgcaatt acccgcgtga g gtg ctg gaa gga ctc 716 Met Leu Glu Gly Leu 50 1 atc ttg gca gtc tcc cgc cag acc aag tca gtc gtc gat att ccc ggt 764 Ile Leu Ala Val Ser Arg Gln Thr Lys Ser Val Val Asp Ile Pro Gly 10 20 55 gca ccg cag cgt tat tct ttc gcg aag gtg tcc gca ccc att gag gtg 812 Ala Pro Gln Arg Tyr Ser Phe Ala Lys Val Ser Ala Pro Ile Glu Val

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25

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5	cct Pro 55								908
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30			_		_	atc Ile		_	_	_	_	_	_		_	_	3308
35						ggc Gly 875											3356
33		_		_	_	gat Asp	_					_				_	3404
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45						cac His											3500
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Asp Gln Thr Ile Asp Lys Ser Thr Glu Arg Pro Leu His Ala Val Lys Val Ile Pro Phe Arg Gly Ala Trp Leu Glu Phe Asp Val Asp Lys Arg Asp Ser Val Gly Val Arg Ile Asp Arg Lys Arg Arg Gln Pro Val Thr Val Leu Leu Lys Ala Leu Gly Trp Thr Thr Glu Gln Ile Thr Glu Arg Phe Gly Phe Ser Glu Ile Met Met Ser Thr Leu Glu Ser Asp Gly Val Ala Asn Thr Asp Glu Ala Leu Leu Glu Ile Tyr Arg Lys Gln Arg Pro Gly Glu Gln Pro Thr Arg Asp Leu Ala Gln Ser Leu Leu Asp Asn Ser Phe Phe Arg Ala Lys Arg Tyr Asp Leu Ala Arg Val Gly Arg Tyr Lys Ile Asn Arg Lys Leu Gly Leu Gly Gly Asp His Asp Gly Leu Met Thr Leu Thr Glu Glu Asp Ile Ala Thr Thr Ile Glu Tyr Leu Val Arg Leu His Ala Gly Glu Arg Val Met Thr Ser Pro Asn Gly Glu Glu Ile Pro Val Glu Thr Asp Asp Ile Asp His Phe Gly Asn Arg Arg Leu Arg Thr Val Gly Glu Leu Ile Gln Asn Gln Val Arg Val Gly Leu Ser Arg Met Glu Arg Val Val Arg Glu Arg Met Thr Thr Gln Asp Ala Glu Ser Ile Thr Pro Thr Ser Leu Ile Asn Val Arg Pro Val Ser Ala Ala Ile Arg Glu Phe Phe Gly Thr Ser Gln Leu Ser Gln Phe Met Val Gln Asn Asn Ser Leu Ser Gly Leu Thr His Lys Arg Arg Leu Ser Ala Leu Gly Pro Gly Gly Leu Ser Arg Glu Arg Ala Gly Ile Glu Val Arg Asp Val His Pro Ser His Tyr Gly Arg Met Cys Pro Ile Glu Thr Pro Glu Gly Pro Asn Ile Gly Leu Ile Gly Ser Leu Ala Ser Tyr Ala Arg Val Asn Pro

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15	Ala	Arg	Ser 115	Pro	Leu	Arg	Arg	Glu 120	Glu	Gly	Ile	Ile	Lys 125	Asn	Ala	

Patent claims

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- An isolated polynucleotide from coryneform bacteria, containing a polynucleotide sequence coding for the rpoB gene, selected from the group
- a) polynucleotide that is at least 70% identical with a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70% identical with the amino acid sequence of SEQ ID No. 2,
 - c) polynucleotide that is complementary to the polynucleotides of a) or b), and
 - d) polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),
 - the polypeptide preferably exhibiting the activity of the β -subunit of RNA polymerase B.
- A polynucleotide as claimed in claim 1, wherein the
 polynucleotide is a DNA, preferably a recombinant DNA,
 that is replicatable in coryneform bacteria.
 - 3. A polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.
- 4. A polynucleotide as claimed in claim 2, containing the nucleic acid sequence as shown in SEQ ID No. 2.
 - 5. A replicatable DNA as claimed in claim 2, containing
 - (i) the nucleotide sequence shown in SEQ ID No. 1, or

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- (ii) at least one sequence that corresponds to sequence (i) within the region of the degeneracy of the genetic code, or
- (iii) at least one sequence that hybridizes with the sequence that is complementary to sequence (i) or (ii), and optionally
 - (iv) sense mutations in (i) that are neutral in terms
 of function.
- 6. A replicatable DNA as claimed in claim 5, wherein the hybridization is carried out under a stringency corresponding to not more than 2x SSC.
 - 7. A polynucleotide sequence as claimed in claim 1, which codes for a polypeptide containing the amino acid sequence shown in SEQ ID No. 2.
- 15 8. A coryneform bacteria in which the rpoB gene is enhanced, especially overexpressed.
 - 9. A process for the production of L-amino acids, especially L-lysine, by fermentation, which process comprises carrying out the following steps:
- a) fermenting the coryneform bacteria that produce the desired L-amino acid, in which bacteria at least the rpoB gene or nucleotide sequences coding therefor are enhanced, especially overexpressed;
 - b) concentrating the L-amino acid in the medium or in the cells of the bacteria, and
 - c) isolating the L-amino acid.
 - 10. A process as claimed in claim 9, wherein there are used bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced.

- 11. A process as claimed in claim 9, wherein there are used bacteria in which at least some of the metabolic pathways that reduce formation of the desired L-amino acid are excluded.
- 5 12. A process as claimed in claim 9, wherein there is used a strain transformed using a plasmid vector, and the plasmid vector carries the nucleotide sequence coding for the rpoB gene.
- 13. A process as claimed in claim 9, wherein expression of the polynucleotide(s) coding for the rpoB gene is enhanced, especially overexpressed.
 - 14. A process as claimed in claim 9, wherein the regulatory/catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide rpoB codes are increased.
 - 15. A process as claimed in claim 9, wherein for the production of L-amino acids there are fermented coryneform microorganisms in which at the same time one or more genes selected from the group
- 20 15.1 the gene dapA coding for dihydrodipicolinate synthase,
 - 15.2 the gene gap coding for glyceraldehyde 3-phosphate dehydrogenase,
- 15.3 the gene tpi coding for triose phosphate isomerase,
 - 15.4 the gene pgk coding for 3-phosphoglycerate kinase,
 - 15.5 the gene zwf coding for glucose-6-phosphate dehydrogenase,
- 30 15.6 the gene pyc coding for pyruvate carboxylase,

- 15.7 the gene mqo coding for malate quinone oxidoreductase,
- 15.8 the gene lysC coding for a feed-back resistant aspartate kinase,
- 5 15.9 the gene lysE coding for lysine export,
 - 15.10 the gene zwal coding for the Zwal protein
 - 15.11 the gene rpsL coding for ribosomal protein S12 are enhanced or overexpressed.
- 16. A process as claimed in claim 9, wherein for the
 production of L-amino acids there are fermented
 coryneform microorganisms in which at the same time one
 or more genes selected from the group
 - 16.1 the gene pck coding for phosphoenol pyruvate carboxykinase,
- 15 16.2 the gene pgi coding for glucose-6-phosphate isomerase,
 - 16.3 the gene poxB coding for pyruvate oxidase
 - 16.4 the gene zwa2 coding for the Zwa2 protein are attenuated.
- 20 17. A coryneform bacteria containing a vector that carries a polynucleotide as claimed in claim 1.
 - 18. A process as claimed in one or more of the preceding claims, wherein microorganisms of the species Corynebacterium glutamicum are used.
- 19. A method of finding RNA, cDNA and DNA, in order to isolate nucleic acids, or polynucleotides or genes, that code for the β -subunit of RNA polymerase B or are

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very similar to the sequence of the rpoB gene, which method comprises using as hybridization probes the polynucleotide containing the polynucleotide sequences as claimed in claims 1, 2, 3 or 4.

- 5 20. A method as claimed in claim 18, wherein arrays, micro arrays or DNA-chips are used.
 - 21. A DNA from coryneform bacteria, coding for β -subunits of RNA polymerase B, wherein the associated amino acid sequences have been altered between positions 1 to 10 in SEQ ID No. 2 by amino acid substitution.
 - 22. A DNA as claimed in claim 21, which DNA codes for β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 5 in SEQ ID No. 2 L-leucine, L-isoleucine or L-valine.
- 15 23. A DNA from coryneform bacteria, coding for β -subunits of RNA polymerase B, wherein the associated amino acid sequences have been altered between positions 190 to 200 in SEQ ID No. 2 by amino acid substitution.
- 24. A DNA as claimed in claim 23, which DNA codes for β 20 subunits of RNA polymerase B, the associated amino acid sequences containing at position 196 in SEQ ID No. 2 Lphenylalanine or L-tyrosine.
 - 25. A DNA from coryneform bacteria, coding for β -subunits of RNA polymerase B, wherein the associated amino acid sequences have been altered between positions 420 to 450 in SEQ ID No. 2 by amino acid substitution.
- 26. A DNA as claimed in claim 25, which DNA codes for the β-subunits of RNA polymerase B, the associated amino acid sequences containing at positions (sic) 439 in SEQ
 30 ID No. 2 any proteinogenic amino acid with the exception of L-histidine.

- 27. A DNA as claimed in claim 25, which DNA codes for the β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 424 in SEQ ID No. 2 L-proline or L-arginine.
- 5 28. A DNA as claimed in claim 25, which DNA codes for the β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 425 in SEQ ID No. (sic) L-threonine or L-alanine.
- 29. A DNA as claimed in claim 25, which DNA codes for the β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 426 in SEQ ID No. 2 L-leucine or L-lysine.
- 30. A DNA as claimed in claim 25, which DNA codes for the β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 429 in SEQ ID No. (sic) L-isoleucine, L-valine or L-leucine.
 - 31. A DNA as claimed in claim 25, which DNA codes for the β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 444 in SEQ ID No. 2 L-leucine, L-tyrosine or L-tryptophan.
 - 32. A DNA as claimed in claim 25, which DNA codes for the β -subunits of RNA polymerase B, the associated amino acid sequences containing at position 446 in SEQ ID No. 2 L-proline or L-isoleucine.
- 33. A DNA from coryneform bacteria, coding for β-subunits of RNA polymerase B, wherein the associated amino acid sequences have been altered simultaneously at one or more positions selected from the group a) position 1 to 10, b) position 190 to 200 and c) position 420 to 450 in SEQ ID No. 2 by amino acid substitution.
 - 34. A DNA as claimed in claim 33, which DNA codes for the β -subunits of RNA polymerase B, the associated amino

acid sequences containing in SEQ ID No. 2 at one or more positions selected from the group a) position 5 L-leucine, b) position 196 L-phenylalanine, c) position 429 L-valine and d) position 439 L-tyrosine.

- 5 35. A DNA as claimed in claim 34, which DNA codes for the β-subunit of RNA polymerase B, the associated amino acid sequence containing L-leucine at position 5, L-phenylalanine at position 196 and L-valine at position 429, shown in SEQ ID No. 4.
- 36. A DNA as claimed in claim 34, which DNA codes for the β -subunit of RNA polymerase B, the associated amino acid sequence containing L-tyrosine at position 439, shown in SEQ ID No. 6.
- 37. A DNA from coryneform bacteria, coding for the βsubunit of RNA polymerase B, wherein the base sequence
 of the DNA contains thymine at position 715, thymine at
 position 1288 and thymine at position 1987, shown in
 SEQ ID No. 3.
- 38 . A DNA from coryneform bacteria, coding for the β subunit of RNA polymerase B, wherein the base sequence of the DNA contains thymine at position 2016, shown in SEQ ID No. 5.

Abstract

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The invention relates to an isolated polynucleotide containing a polynucleotide sequence selected from the group

- 5 a) polynucleotide that is at least 70% identical with a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70% identical with the amino acid sequence of SEQ ID No. 2,
 - c) polynucleotide that is complementary to the polynucleotides of a) or b), and
 - d) polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),

and to a process for the production of L-amino acids by fermentation using coryneform bacteria in which at least the rpoB gene is present in enhanced form, and to the use of polynucleotides containing the sequences of the invention as hybridization probes.